AET AND RADIATION-INDUCED CROSSING-OVER IN MALE DROSOPHILA MELANOGASTER [1]

SIDNEY MITTLER

Department of Biological Sciences, Northern Illinois University, De Kalb, Illinois 60115

AET, S, 2-aminoethylisothiouronium Br, has reported to be one of the most efficient sulfhydryl compounds that will protect mice against death from radiation (Doherty and Burnett, 1955). The various hypotheses suggested for the mechanism of protection include those that should protect chromosomes from radiation-induced damage. Induced crossing-over in male *Drosophila,* or more properly "pseudo-crossing-over" to indicate induced breakage, was one of the radiation-induced chromosomal aberrations selected to test whether AET would protect against this effect. Lefevre (1947) with work on radiation-induced somatic crossing-over, and Parker (1948) and Whittinghill (1951) from evidence with induced crossing-over in the male *Drosophila* interpreted this as a result of chromosomal breakage. Herskowitz and Abrahamson (1957), Olivieri and Olivieri (1964) related dosage to amount of induced crossovers and concluded that a "two-hit" phenomenon was responsible.

Materials and Methods

Adult male *Drosophila melanogaster* 2–16 hours old, heterozygous for third chromosome roughoid (*ru,* 0.0), hairy (*h,* 26.5), thread (*th,* 43.2), scarlet (*st,* 44.0), curled (*cu,* 50.0), stripe (*sr,* 62.0), ebony (e^s, 70.7), claret (*ca,* 100.7), as a result of cross between Oregon-R and "rucuca," were injected with 3 mg. of AET/ml. in 0.01 *M* NaOH. The AET was converted to effective MEG with a pH of 7.2. The injection was done with a glass needle with a tip diameter of 0.06 mm. The amount of fluid introduced between the dorsal third and fourth tergites was determined by increase in the weight of the flies and was found to be approximately 0.1 μl. or 3.33×10^{-5} mg. of AET per fly. The *Drosophila* males, usually in two groups of 25, one a control group injected with saline and the other treated with AET, were exposed immediately after injections to 2000 r of x-ray radiation in air by a Mattern x-ray unit with a Thermax tube at 100 KV, 5 ma., $\frac{1}{2}$ mm. of Al filter, 320 r/min. (determined by a Victoreen Model 570 condenser r-meter) at a distance of 11 cm.

In one series, 120 pretreated AET males and 101 control males were irradiated and allowed to recover in air and then mass-mated to virgin "rucuca" at a ratio of one male to three females, aged two to three days. The males were presented with a virgin group of females every three days. On the ninth day after irradiation, each male was isolated in a small vial and given three females, and on the 12th day this was repeated in a new vial of food. Thus, the offspring of each male could be recorded for 9–12- and 12–15-day broods after irradiation.

[1] Supported by Contract No. AT(11-1) 1081 with U. S. Atomic Energy Commission.

In another series 212 males were injected with AET and a control group of 125 were exposed to 2000 r in air. Then they were both post-treated with flowing N_2 for 30 minutes. The flies were mated at a ratio of one male to three females in broods 0–3 day, 3–6 day and then mated daily on days 6 through 15. All experiments were kept at 24° C. The design of the experiments was based on the consensus of several authors (Auerbach, 1954; Bateman, 1956; Savhagen, 1961) that the cells in meiosis at the time of radiation of young adult males' testes will be mature usable spermatozoa 7–9 days later. Thus, induced crossing-over in diploid cells should appear in the offspring resulting from matings 7 or 8 days after irradiation. Crossovers as suggested by Auerbach (1954) were scored when at least two or more mutants (r_2) were present, for presence or absence of a single mutant (r_1) could represent a deletion or mutation.

TABLE I

Radiation-induced crossovers in adult males 2–16 hours old, heterozygous for ru h th st cu sr e^s ca, injected with 3 mg./ml. of AET, and then irradiated with 2000 r of x-rays and recovered in air.

Brood	AET				Control			
	Crossover		Total	r_2% Total gametes	Crossover		Total	r_2% Total gametes
	r_1	r_2			r_1	r_2		
9–12-day	7	50	8509	0.588%*	8	45	5078	0.886%
12–15-day	13	41	11415	0.359%	10	19	4187	0.453%

* $\chi^2 = 4.02$.

RESULTS

The males treated with AET and then irradiated had a significant decrease in the number of r_2-type crossovers in the 9- to 12-day brood as compared to offspring from the control males (Table I). By means of a 2×2 contingency table comparing crossovers to total offspring the chi square was calculated to be 4.02. However, there was no significant difference between the 12–15-day brood of the pretreated AET males and control males. Another method of presenting the data, namely the percentage of males induced to produce crossovers, was considered. Although this method would avoid to some extent the problem of clusters, the difficulty would be in giving equal status to males with considerable differences in the number of their offspring. Thirty-nine out of 118 AET-treated (33.1%) males produced crossovers in the 9–12-day brood, while 25 out of a total 87 fertile control males (28.7%) had crossovers in their offspring. In the 12–15-day brood there were 17.4% and 16.6% males, respectively, induced to produce crossovers. Although the 12–15-day brood represents spermatogonia that may have undergone further divisions, and thus produced clusters, the number of crossovers per thousand flies (gametes) was less in this brood in both the AET and control offspring than in 9–12-day brood. Non-crossovers are also produced in clusters.

The analysis of the induced crossovers as to the region in which breakages occurred (if it is indeed the case) revealed that the majority of them were in region

TABLE II

Location and number of breaks in radiation-induced (2000 r) crossovers (r_2) in males whose third chromosome was heterozygous for ru h th st cu sr e^s ca. Radiation in air and recovery in air. Location of regions of breakage: ru 1 *h* II *th* III *st* IV *cu* V *sr* VI e^s VII *ca*

Region	Number of breaks			
	AET (3 mg./ml.)		Control	
	9–12-day	12–15-day	9–12-day	12–15-day
I	1			
II				
III			1	
IV	43	40	41	19
V	5	1	2	
VI	1	1	3	
VII	1		5	
Non-crossover	8452	11361	5025	4158

IV between *st* and *cu* and included the centromere (Table II). AET did not influence the region of the breakage, for in the control 9–12-day brood, 80.3% of the breaks occur in this centromere region, while in the offspring of AET-treated males, 84.3% of all breaks occur in this same region. The 12–15-day broods had a largest percentage of breaks about the centromere.

The series on nitrogen post-treated was conducted to test the hypothesis that the AET affected the recovery system. The data in Table III do indicate that post-treatments in nitrogen tend to reduce the number of crossovers produced by

TABLE III

Radiation-induced crossovers in adult males 2–16 hours old heterozygous for ru h th st cu sr e^s ca' injected with 3 mg./ml. of AET irradiated with 2000 r in air and post-treated with N_2 for 30 minutes

Brood	AET				Brood	Control			
	Crossover		Total	r_2% Total gametes		Crossover		Total	r_2% Total gametes
	r_1	r_2				r_1	r_2		
day 6	1	0	1010		day 6	1	0	1328	
day 7	1	3	672	0.446	day 7	1	1	841	0.119
day 8	0	2	736	0.272	day 8	1	5	1274	0.392
day 9	1	17	2643	0.681	day 9	0	19	2582	0.736
day 10	2	14	2669	0.525	day 10	3	13	1459	0.891
day 11	1	14	3010	0.465	day 11	3	13	2182	0.596
day 12	4	21	3376	0.622	day 12	0	2	2560	0.078
day 13	1	3	3358	0.089	day 13	8	6	2432	0.247
day 14	0	18	2303	0.782	day 14	0	9	1403	0.641
day 15	0	1	1246	0.080	day 15	0	4	1249	0.320

the control males so that there is no significant difference between 9–12-day brood of control males and that of AET and post-treated nitrogen males with respect to the number of crossovers. Although the data were presented of the daily broods, the three daily broods of days 9, 10, and 11 were totaled and then compared to the 9–12-day broods of flies recovered in air. The daily broods of days 12, 13, and 14 were also totaled and compared to 12–15-day broods of flies recovered in air. By this method the daily brood data presented in Table III were analyzed and the 9–12-day brood of control males treated in N_2 produced 45 (0.723%) crossovers out of a total of 6225. This is lower than 0.886% crossovers in the corresponding brood recovered in air (Table I). The 9–12-day brood of AET and post-treated

TABLE IV

Location and number of breaks in radiation-induced (2000 r) crossovers (r_2) in males whose third chromosome was heterozygous for ru h th st cu sr e^s ca. Radiation in air, post-treated with nitrogen for 30 minutes. Location of regions of breakage: ru I *h* II *th* III *st* IV *cu* V *sr* VI e^s *VII ca*

Brood-day	AET (3 mg./ml.)		Control	
	Number of breaks, region	Total gametes	Number of breaks, region	Total gametes
6	0	1009	0	1327
7	1 I, 1 IV, 2 V, 1 VIII	668	1 I, 1 III, 1 V, 1 VII	839
8	2 IV	734	5 IV	1268
9	17 IV	2625	18 IV, 1 V	2563
10	13 IV, 1 VI,	2653	12 IV, 1 V	1443
11	1 I, 1 III, 12 IV, 1 V, 1 VII	2995	1 III, 13 IV, 1 VI	2169
12	21 IV	3351	2 IV	2558
13	3 IV	3354	6 IV	2418
14	9 IV	2285	18 IV	2285
15	4 IV	1123	1 IV	1245

N_2 males yielded 45 (0.541%) crossovers in a total of 8322 which is quite similar to 50 (0.588%) crossovers in a total of 8509 in the 9–12-day brood of AET and air-recovered males. The control 12–15-day brood as a result of post-treatment with N_2 had 17 crossovers in 6395 (0.266%) while the same brood treated with AET and post-treated with nitrogen, 42/9037 (0.456%) which is chi square difference of 3.85. The nitrogen post-treatment reduced the amount of crossovers in control 12–15-day brood but not the percentage of crossovers in the broods of males treated with AET. The region of the breakage was not affected by the post-treatment of N_2 (Table IV) and the breakage was most frequent about the centromere.

DISCUSSION

The x-ray-induced crossing-over in young adult male *Drosophila* resulted in a greater percentage of crossovers in the older spermatogonia (those nearer to meiosis) than in the younger spermatogonia. The older spermatogonia represented by 9–12-day brood indeed may be a more sensitive period for induced crossing-over.

It is also possible that young spermatogonia were more susceptible to injury and hence failed to survive, and thus there were fewer crossovers. There is also a relatively smaller population of spermatogonia represented by 12–15-day brood at the time of radiation. Olivieri and Olivieri (1964) also found that radiation induced more crossovers in the late spermatogonia than the early spermatogonia.

The question raised by the data in Table I is whether AET actually protects the 9–12-day brood from crossing-over, or whether the broken or damaged chromosomes are prevented from repairing or rejoining, hence are lost. From the evidence in the literature AET and other sulfhydryls apparently do not give protection to radiation-induced chromosomal aberrations in *Drosphila*. Edington (1958) reported that when AET was used as a possible protector against radiation-induced mutations in male *Drosophila,* there was an increase in "dominant lethals" and in the numbers of sex-linked lethals (Kaplan and Lyon, 1953). Mittler (1964) found that pretreatment with MEA, AET, and glutathione did not protect the *Drosophila* testes from radiation-induced sex-linked lethals, translocations, deletions of the X chromosome, loss of X or Y chromosomes and "dominant lethals," and that glutathione and MEA increased the number of "dominant lethals" and loss of chromosomes in the XO method. Rugh and Fu (1965) reported that AET did not protect the cytoplasm or nucleus of the haploid *Arbacia* cell from gamma rays.

The region that includes the centromere was found to have the majority of the breakages or exchanges that occurred. Olivieri and Olivieri (1964), Friesen (1937), Parker (1948) and Whittinghill (1951) also reported that induced crossing-over is greatest in this centromeric heterochromatic region. AET or post-treatment with N_2 did not influence the breakage in this region.

The paradox of a radioprotective substance actually increasing radiation damage may be explained by AET having a greater adverse effect on the recovery process than on actual protection, if any, against the initial effect. It may be that 2000 r may so overwhelm the initial protective effect of AET, if any, that only the effect on the recovery system is tested. For this reason, the series on nitrogen post-treatment was conducted. If one assumes that AET induces anoxia or by some means prevents the recovery of broken chromosomes, then control males whose testes were injured by radiation in air and who were then subjected to nitrogen should yield data similar to AET-treated males recovered in air and thus have less crossovers.

The data in Table III do indicate that post-treatment with nitrogen tended to reduce the number of crossovers produced by the control males so that there is no significant difference between 9–12-day brood of control males and that of AET-treated males with respect to the number of crossovers. Since post-treatment with N_2 does induce anoxia and since the nitrogen post-treatment has no effect on AET-treated males with respect to a change in the number of crossovers, it appears as if AET injection induces an anoxia condition which simulates to some extent the post-nitrogen treatment.

Oliveri and Oliveri (1964) were surprised to find an increase in crossing-over in males irradiated in air and post-treated in N_2, for they had also noted that N_2 when given between the fractions restored the frequency of the induced crossing-over as if it were unfractionated, and that N_2 acted by preventing the restitution of

breaks. The data presented in this paper indicated that N_2 post-treatment tends to reduce the number of crossovers and that the day 10 brood used only by the Olivieris to test the effect of post-treatment of N_2 produced the largest percentage of crossovers in our control group post-treated with nitrogen (Table III). The young spermatogonia, broods day 12–15, were definitely affected by the post-treatment of N_2. The 30 minutes of N_2 treatment were sufficient to decrease the number of crossovers, probably by keeping the broken chromatids or chromosomes from uniting with a sister pair, hence less crossovers. If the broken or injured chromosomes are lost as a result of the N_2 treatment, this would also result in a decrease in radiation-induced crossovers. However, AET does not affect the younger spermatogonia, for the decrease in crossovers was in the older spermatogonia. It is indeed possible that something besides anoxia induced by AET may be interfering with the recovery process. Sobels (1963) pointed out the extreme difference in response of the mature spermatozoa compared to that of spermatids with respect to N_2 post-treatments. Not only are there differences in radiosensitivity for the various pre- and post-treatments. Even a difference of several hours in the age of mature sperm can result in significant differences in radiation-induced lethals (Lefevre and Jonsonn, 1964).

AET does not protect against breakage of chromosomes or whatever radiation-induced damage causes crossing-over in male *Drosophila*. Although there are fewer induced crossovers in the 9–12-day brood which represents cells about to go into meiosis, the AET effect is eliminated by post-treatment with N_2. The induced crossovers of N_2 post-treated males, whether injected with AET or saline, will be lower as a result of the N_2 treatment.

The excellent technical assistance of Raymond U is acknowledged.

Summary

1. *Drosophila melanogaster* males heterozygous for *ru h th st cu sr* e^s *ca* were injected with AET and irradiated with 2000 r and backcrossed to "rucuca." The number of induced crossovers with two or more mutants (r_2) was significantly decreased in the 9–12-day broods of males treated with AET as compared to controls.

2. The hypothesis that AET interfered with a recovery process probably by an induced anoxia was tested by N_2-treatment after the x-ray-treatments. The N_2 post-treatments had no effect on AET-treated and irradiated males with respect to crossovers; however, the young spermatogonia represented by brood day 12–15 of the control males were induced by the N_2 treatment to produce less crossovers.

3. Since the AET effect is eliminated by N_2 post-treatment, an anoxia production by AET can be a possible mechanism for reduction of crossovers. Nitrogen post-treatment tends to reduce the number of radiation-induced crossovers. AET did not affect the young spermatogonia.

4. The induced breakage occurred primarily between regions *st* and *cu* and thus in or about the centromeric region. AET has no influence on the region of the breakage.

5. Crossovers were found to be more frequent in the 9–12-day brood than in the 12–15-day brood.

LITERATURE CITED

AUERBACH, C., 1954. Sensitivity of the *Drosophila* testis to the mutagenic action of x-rays. *Zeitschr. Induktive Abstammungs-Vererbungslehre,* **86**: 113–125.

BATEMAN, A. J., 1956. Mutagenic sensitivity of maturing *Drosophila* sperm, I. Dominant lethals. *J. Genetics,* **54**: 400–410.

DOHERTY, D. G., AND W. T. BURNETT, JR., 1955. The protective effect of S B-aminoethylisothiuronium·Br·HBr and related compounds against x-radiation death in mice. *Proc. Soc. Exp. Biol. Med.,* **89**: 312–314.

EDINGTON, C. W., 1958. The effect of S, 2-aminoethylisothiuronium bromide hydrobromide (AET) on the induction of dominant and sex-linked recessive lethals in *Drosophila melanogaster. Amer. Nat.,* **92**: 371–374.

FRIESEN, H., 1937. Mechanism of crossing-over in males of *Drosophila melanogaster. J. Genetics,* **35**: 141–150.

HERSKOWITZ, I. H., AND S. ABRAHAMSON, 1957. Induced changes in female germ cells of *Drosophila.* IV. Dependence of induced crossover-like exchanges in oocytes and oogonia upon x-ray intensity. *Genetics,* **42**: 444–453.

KAPLAN, W. D., AND M. F. LYON, 1953. Failure of mercaptoethylamine to protect against the mutagenic effects of radiation. I. Experiments with *Drosophila. Science,* **118**: 776–777.

LEFEVRE, G., JR., 1947. The relative effectiveness of fast neutrons and gamma rays in producing somatic crossing-over in *Drosophila. Genetics,* **32**: 33.

LEFEVRE, G., JR., AND U.-B. JONSONN, 1964. X-ray induced mutability in male germ cells of *Drosophila melanogaster. Mutation Research,* **1**: 231–246.

MITTLER, S., 1964. The failure of sulfhydryl compounds, AET, MEA, and glutathione to protect against x-ray induced chromosome aberrations in male *Drosophila. Int. J. Rad. Biol.,* **8**: 405–413.

OLIVIERI, G., AND A. OLIVIERI, 1964. Evidence for the two-hit nature of x-ray induced crossing-over in the centromeric region of *Drosophila* males. *Mutation Research,* **1**: 279–295.

PARKER, D. R., 1948. Observations on cross-over induced by x-rays in the males of *Drosophila. Genetics,* **33**: 304–310.

RUGH, R., AND K. FU, 1965. AET as a radioprotective agent at the cellular level. *Biol. Bull.,* **128**: 125–128.

SAVHAGEN, R., 1961. The frequency of XO males and induced autosomal crossovers after irradiation of *Drosophila melanogaster* males in air or commercial nitrogen. *Hereditas,* **47**: 23–42.

SOBELS, F. H., 1963. Radiosensitivity and repair in different germ cell stages of *Drosophila. Proc. 11th Intern. Congr. Genet., The Hague,* **2**: 235–255.

WHITTINGHILL, M., 1951. Some effects of gamma rays on recombination and on crossing-over in *Drosophila melanogaster. Genetics,* **42**: 332–355.